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Article (Published Version)

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Mol. Cell. Biol. **2003, 23(18):6553. DOI: 10.1128/MCB.23.18.6553-6563.2003.**

MOLECULAR AND CELLULAR BIOLOGY, Sept. 2003, p. 6553–6563 Vol. 23, No. 18
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.18.6553–6563.2003

Molecular Characterization of the *Schizosaccharomyces pombe nbs1*$^+$ Gene Involved in DNA Repair and Telomere Maintenance

Masaru Ueno,[1]* Tomofumi Nakazaki,[2] Yufuko Akamatsu,[2,3] Kikuo Watanabe,[1] Kazunori Tomita,[1] Howard D. Lindsay,[4] Hideo Shinagawa,[3] and Hiroshi Iwasaki[2]

Department of Chemistry, Faculty of Science, Shizuoka University, Shizuoka 422-8529,[1] Graduate School of Integrated Science, Yokohama City University, Tsurumi-ku, Yokohama, Kanagawa 230-0045,[2] and Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871,[3] Japan, and Genome Damage and Stability Centre, University of Sussex, Brighton BN1 9RQ, United Kingdom [4]

Received 10 April 2003/Returned for modification 17 May 2003/Accepted 6 June 2003

The human MRN complex is a multisubunit nuclease that is composed of Mre11, Rad50, and Nbs1 and is involved in homologous recombination and DNA damage checkpoints. Mutations of the MRN genes cause genetic disorders such as Nijmegen breakage syndrome. Here we identified a *Schizosaccharomyces pombe nbs1*$^+$ homologue by screening for mutants with mutations that caused methyl methanesulfonate (MMS) sensitivity and were synthetically lethal with the *rad2*Δ mutation. Nbs1 physically interacts with the C-terminal half of Rad32, the *Schizosaccharomyces pombe* Mre11 homologue, in a yeast two-hybrid assay. *nbs1* mutants showed sensitivities to γ-rays, UV, MMS, and hydroxyurea and displayed telomere shortening similar to the characteristics of *rad32* and *rad50* mutants. *nbs1*, *rad32*, and *rad50* mutant cells were elongated and exhibited abnormal nuclear morphology. These findings indicate that *S. pombe* Nbs1 forms a complex with Rad32-Rad50 and is required for homologous recombination repair, telomere length regulation, and the maintenance of chromatin structure. Amino acid sequence features and some characteristics of the DNA repair function suggest that the *S. pombe* Rad32-Rad50-Nbs1 complex has functional similarity to the corresponding MRN complexes of higher eukaryotes. Therefore, *S. pombe* Nbs1 will provide an additional model system for studying the molecular function of the MRN complex associated with genetic diseases.

Nijmegen breakage syndrome is an autosomal recessive genetic disease characterized by developmental defects, microcephaly, immune deficiency, and a high incidence of cancer (8, 38, 64). Cells from patients with Nijmegen breakage syndrome show genetic instability and hypersensitivity to γ-ray irradiation and are impaired in cellular responses to γ-ray irradiation, including radio-resistant DNA synthesis (29, 52, 57). These cytogenetic features are indistinguishable from those of another genetic disease, ataxia telangiectasia (50). The cellular defects in Nijmegen breakage syndrome and ataxia telangiectasia cells are suggested to be caused by defective responses to DNA double-strand breaks (DSBs) due to a mutation of the responsible genes, *NBS1* and *ATM*, respectively.

DSBs are not only generated by exogenous DNA-damaging agents such as γ-ray irradiation; they also occur during normal DNA replication. There are two main pathways for the repair of such DSBs: nonhomologous end joining and homologous recombination. The Mre11-Rad50-Xrs2 protein complex (MRX complex) from *Saccharomyces cerevisiae* has been suggested to be involved in the initial steps of both repair pathways (24). In nonhomologous end joining, the MRX complex is suggested to stimulate intermolecular DNA end joining by DNA ligase IV (11). In homologous recombination, the processing of DSB ends to produce 3′ single-stranded tails is a very important reaction that provides substrates for homologous pairing and strand exchange reactions. From early studies, the MRX complex was thought to function as a nuclease that is involved in such DNA end processing (24). However, recent studies suggest a structural role of the MRX complex in holding DNA ends and/or sister chromatids together (12, 17, 25, 26, 28, 30).

The Mre11 and Rad50 proteins are highly conserved from *S. cerevisiae* to humans. However, Xrs2 is not well conserved, and the functional counterpart of Xrs2 is considered to be Nbs1 in vertebrates. The similarity of the overall sequences between those two proteins is very weak and is limited to the N-terminal forkhead-associated domain and a small C-terminal region (56). Although Nbs1 contains a BRCA1 C-terminal (BRCT) domain in the N-terminal region, Xrs2 does not contain this domain. Nbs1 binds to the Mre11 subunit of the Rad50-Mre11 complex via the C-terminal conserved region (18, 56).

The MRX(N) complex possesses exo- and endonuclease activity in vitro (21, 49, 59, 63). Mre11 contains the phosphodiesterase motif responsible for the nuclease activity (27). Rad50 is related to the SMC proteins, which contain Walker A and B motifs, responsible for ATPase activity, separated by a long coiled-coil region (26, 27). Rad50 stimulates the nuclease activity of yeast and human Mre11 (48, 60). The biochemical functions of Xrs2 and Nbs1 during the recombination process remain unclear. To date, the only biochemical activity assigned to Nbs1 is stimulation of the unwinding and hairpin cleavage activities of the human Mre11-Rad50 complex (49). The yeast Mre11-Rad50 complex cleaves hairpin structures in the absence of Xrs2 (59). Surprisingly, some mutations that eliminate the nuclease activity of yeast Mre11 do not confer telomere defects or strong DNA repair deficiency (33, 41). Therefore, it

* Corresponding author. Mailing address: Department of Chemistry, Faculty of Science, Shizuoka University, 836 Oya, Shizuoka 422-8529, Japan. Phone: 81 54 238 4762. Fax: 81 54 237 3384. E-mail: scmueno@ipc.shizuoka.ac.jp.

seems likely that end processing is not the major role of the complex.

Mutations in the genes that encode the components of the MRX(N) complex result in DNA damage sensitivity, genome instability, telomere shortening, and aberrant meiosis (14). These phenotypes are considered to be related to deficiencies in DNA repair abilities involving the DNA-processing activity of the MRX(N) complex. In addition, recent studies have shown other functions for the Mre11 complex in checkpoint signaling and DNA replication (6, 31, 37, 39, 68). Hypomorphic mutations in the human *MRE11* gene cause ataxia telangiectasia-like disorder, whose symptoms are very similar to those of Nijmegen breakage syndrome and ataxia telangiectasia (51). In the cells from these patients, DNA synthesis is not arrested in response to ionizing radiation. Yeast mutants with mutations of *mre11*, *rad50*, or *xrs2* are hypersensitive to hydroxyurea and show no delay in DNA synthesis in the presence of hydroxyurea or bleomycin, indicating a defect in the intra-S checkpoint (15, 23). Mre11 and Xrs2/Nbs1 have been shown to be phosphorylated in response to ionizing radiation, and this phosphorylation is dependent on checkpoint kinase function (22, 34, 70).

In the fission yeast *Schizosaccharomyces pombe*, which is distantly related to *S. cerevisiae*, the *rad32*$^+$ gene was first identified as a gene corresponding to a mutant that showed hypersensitivity to DNA damage and was later found to be the structural and functional homologue of *MRE11* (58, 65, 66). The *rad50*$^+$ gene in *S. pombe* was identified through its sequence similarity with the *S. cerevisiae*, *Caenorhabditis elegans*, and human *RAD50* genes. Both *rad32*$^+$ and *rad50*$^+$ are required for DNA repair and telomere maintenance (25). However, no Nbs1/Xrs2 homologue in *S. pombe* has been reported; one of the reasons for this is that no structural homologue can be found in the database even though the whole genome sequence of *S. pombe* has been determined (67).

To identify novel genes involved in homologous recombination and recombination repair in *S. pombe*, we previously isolated seven *slr* mutants, whose growth was dependent on the *rad2*$^+$ function (*slr* stands for synthetic lethality with *rad2*) (62). The *rad2*$^+$ gene encodes a structure-specific endonuclease homologous to mammalian Fen-1 that is required for Okazaki fragment maturation (46). Yeast cells defective in Fen-1 nuclease activity are nonviable in combination with mutations that inactivate homologous recombination (16, 54). Thus, *slr* mutants are often considered to be defective in homologous recombination and recombination repair. Indeed, *S. pombe rhp51* (*RAD51* homologue), *rhp54* (*RAD54* homologue), *rhp57* (*RAD57* homologue), *rad50*, and *rad32* mutations are lethal when combined with the *rad2* mutation (25, 45, 58, 62). In an earlier study, we characterized two genes, *rhp57*$^+$ and *rad60*$^+$, identified among the seven *slr* mutants. The former is a structural and functional homologue of *S. cerevisiae RAD57*, and the latter is a novel recombination gene for which no structural homologue has been found in the database (43, 62).

Since *slr* screening is an effective mechanism for isolating recombination-related genes, we performed a second large-scale screening and obtained further *slr* candidates. In this study, we characterized the eighth *slr* mutation, *slr8*. DNA sequencing of complementing plasmids revealed that *slr8*$^+$ encodes a protein with a forkhead-associated domain in the N-terminal region and an Mre11-binding consensus sequence in the C-terminal region. Indeed, Slr8 protein was found to interact physically with Rad32 via the C-terminal region of Rad32 in a yeast two-hybrid assay. *slr8*Δ mutants showed DNA repair defect phenotypes very similar to those of *rad32*Δ and *rad50*Δ mutants and showed epistatic interactions with these mutations. Although the overall sequence similarities of the three proteins, *S. cerevisiae* Xrs2 and Slr8 and vertebrate Nbs1, are limited, our data allow us to conclude that *slr8*$^+$ is a functional homologue of *XRS2*/*NBS1*, and thus we have designated it *nbs1*$^+$ in *S. pombe*.

MATERIALS AND METHODS

***S. pombe* strains, media, and genetic methods.** The *S. pombe* strains used in this study are listed in Table 1. All strains are derivatives of 972 h^-, 975 h^+, or JY741 h^-. Standard procedures and media were used for propagation and genetic manipulation (42). To measure the sensitivity of cells to γ-rays or UV light, exponentially growing cells were irradiated with γ-rays from a ^{60}Co source at a dose rate of 100 to 200 Gy/h or with UV light from a germicidal lamp (UVP UV-Crosslinker, CL-1000) at a dose rate of 50 to 100 J/m^2/min. Duplicates of irradiated cells and unirradiated cells were plated on YPAD medium (1% yeast extract, 2% polypeptone, 2% glucose, 20 μg of adenine per ml) and incubated at 30°C for 4 days, and the colonies were counted. For semiquantitative analysis of DNA repair activity, the spot assay was employed as described previously (62). Briefly, 3 μl of 10-fold dilutions of log-phase cells (0.5×10^7 cells/ml) were spotted onto a YPAD (2% agar) plate or YPAD plate containing the indicated concentration of methylmethane sulfonate (MMS) or hydroxyurea. All experiments were repeated at least twice and gave similar results.

***slr* mutant isolation.** *S. pombe* strain SP184 (*h$^-$ smt-0 ade6-704 ura4-D18 leu1-32 rad2*::*ura4*) carrying pAUR2, which is a derivative of pUR18 that carries the *rad2*$^+$ gene and *ura4*$^+$ as a selective marker (62), was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine as described by Moreno et al. (42). Initially, approximately 100,000 colonies were roughly examined for the plasmid dependency of their growth by replica plating on Edinburgh minimal medium (EMM) plates containing 0.1% 5-fluoroorotic acid, and 521 5-fluoroorotic acid-sensitive colonies were isolated. These isolates were examined for MMS sensitivity by replica plating on EMM plates containing 0.004% MMS, and 92 MMS-sensitive colonies were obtained. Their 5-fluoroorotic acid sensitivity was confirmed by a spot assay on EMM plates containing 5-fluoroorotic acid, and 42 of the isolates were found to be sensitive to both MMS and 5-fluoroorotic acid.

In this study, we analyzed 15 of the isolates that showed much higher sensitivity to MMS than the others. These 15 isolates were then crossed with SP185 (*h$^+$ ade6-704 ura4-D18 rad2*::*ura4*) carrying pAUR2. Three isolates were found to produce progeny with the phenotypes of MMS sensitivity and 5-fluoroorotic acid resistance, indicating that the growth of the three strains was not dependent on pAUR2. The remaining 12 strains were backcrossed three times with the wild-type strain, and the final progeny that were obtained had the genotype *slr rad2*$^+$ *ade ura leu*.

Cloning of *slr* genes. Each of the new *slr* mutants was transformed with the *S. pombe* genomic libraries constructed in the laboratories of H. Masukata (Osaka University) and A. M. Carr (University of Sussex) and spread on EMM plates containing appropriate supplements and MMS (0.004%). The transformants were examined for plasmid-dependent MMS resistance, and the plasmids which complemented the MMS sensitivity were recovered as described previously (43).

***slr8* cDNA cloning.** The cDNA corresponding to the *slr8*$^+$ N-terminal region was amplified by PCR with a sense primer, IWA 252 (5′-CTCATATGAACAG ACAAGCTGGGTCCAG-3′), and an antisense primer, IWA184 (5′-GAGGAT CCTTAAAAGTGAAACTTGAGATCATTAAATTCATCG-3′), with the *S. pombe* cDNA library (Clontech) as the template. The PCR product was cloned into the *Nde*I and *Bam*HI sites of the vector pBSNde, giving plasmid pNT139. pBSNde was constructed by insertion of an *Nde*I linker (5′-CCCATATGGG-3′) into the *Hin*cII site of pBluescript II SK (+). The cDNA corresponding to the C-terminal region was amplified by PCR with a sense primer, IWA 256 (5′-CT CCATATGTGGATAATTGAGGCTGAGGCTGAGGGTGACATTC-3′), and an antisense primer, IWA37 (5′-CAGAGTCATATACTGCGTTG-3′), and the product was cloned into the *Nde*I and *Bam*HI sites of pBSNde, giving plasmid pNT136.

The full-length open reading frame (ORF) of the cDNA was constructed by insertion of the *Nde*I-*Eco*RV fragment encoding the N-terminal portion of *slr8*$^+$ from pNT139 into the *Nde*I and *Eco*RV sites of pNT136, giving pNT140. The

TABLE 1. *S. pombe* strains used in this study

Strain	Genotype	Source[a]
142	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad32*::*ura4*$^+$	A. Matsuura
968	*h*^{90}	NCYC
972	*h*$^-$	NCYC
975	*h*$^+$	NCYC
B54	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 rhp51*::*his3*$^+$	This study
JY741	*h*$^-$ *leu1-32 ura4-D18 ade6-M216*	M. Yamamoto
KT120	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad50*::*LEU2*	Our lab stock
KW015	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 ade6-M210 nbs1*::*ura4*$^+$ *rad50*::*LEU2*	This study
SP154	*h*$^+$ *ura4-D18*	NCYC
SP184	*h*$^-$ *smt-0 ade6-704 leu1-32 ura4-D18 rad2*::*ura4*/*pAUR2*	This study
SP185	*h*$^+$ *ade6-704 ura4-D18 rad2*::*ura4*/*pAUR2*	This study
SPN100	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 nbs1*::*ura4*$^+$	This study
SPN103	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 nbs1*::*ura4*$^+$ *rhp51*::*his3*$^+$	This study
SPN114	*h*$^+$ *mat1PD*::*LEU2 leu1-32 ura4-D18 his3-D1 arg3-D1*	Our lab stock
SPN124	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1*	Our lab stock
SPN138	*h*$^+$ *mat1PD*::*LEU2 leu1-32 ura4-D18 his3-D1 arg3-D1 nbs1*::*ura4*$^+$	This study
SPN141	*h*$^+$ *mat1PD*::*LEU2 leu1-32 ura4-D18 his3-D1 arg3-D1 rad32*::*ura4*$^+$	This study
SPN148	*h*$^+$ *mat1PD*::*LEU2 leu1-32 ura4-D18 his3-D1 arg3-D1 rad50*::*ura4*$^+$	This study
YA137	*h*$^+$ *mat1PD*::*LEU2 ura4-D18 leu1-32 rad2*::*ura4*$^+$	This study
Rad3D	*h*$^-$ *ura4-D18 leu1-32 ade6-704 rad3*::*ura4*$^+$	A. Carr

[a] NCYC, National Collection of Yeast Cultures.

nucleotide sequence of the full-length ORF was determined with an automated DNA sequencer (ABI Prism 3100) and shown to match SPBC6B1.09c in cosmid c6B1. The exons predicted at the Sanger Centre database (http://srs.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-e+[{EMBL%20EMBLNEW}-acc:AL021838]) and the exons predicted by our cDNA sequencing are shown below. Exons predicted in the Sanger Center database were complement(26318 to 26354), complement (26205 to 26258), complement(26078 to 26166), complement(25825 to 25988), complement(24495 to 25785), and complement(24185 to 24448); exons predicted by our cDNA sequencing were complement(26318 to 26354), complement(26205 to 26258), complement(26078 to 26166), complement(25825 to 25973), complement(25703 to 25785), complement(24495 to 25660), and complement(24185 to 24448). The numbers used above correspond to the numbering used in cosmid c6B1 in the Sanger Centre database. The cDNA sequence data are available from the DDBJ/EMBL/GenBank nucleotide databases (accession no. AB099299).

3′- and 5′-RACE. Total RNA was prepared from wild-type *S. pombe* strain 968 (*h*^{90}) with an RNeasy mini kit (Qiagen), and then the mRNA was purified with an Oligotex-dT30 Super mRNA purification kit (TaKaRa). 3′-rapid amplification of cDNA ends (RACE) and 5′-RACE experiments were carried out with a 3′-Full RACE core set (TaKaRa) and 5′-Full RACE core set (TaKaRa), respectively, according to the manufacturer's instructions. The primer sets used for 3′-RACE and 5′-RACE were as follows: IWA328 (sense primer for 3′-RACE), GTCATCCGAGAAATCGAATGCTAACAGTA; IWA318 (reverse transcription primer for 5′-RACE), CGTATCGAGGTCCTTTAC; IWA321 (first sense primer for 5′-RACE), GCCATGCTCGTTTTACG; IWA320 (first antisense primer for 5′-RACE), CGAATCGTCAGATACATTTC; IWA322 (second sense primer for 5′-RACE), GTGAGAAAGACTACTTTACC; and IWA319 (second antisense primer for 5′-RACE), CCTACAATATAAGTTCCTGG.

Yeast two-hybrid analysis. Gal4-based Matchmaker Two-Hybrid System 3 (Clontech) was used for the yeast two-hybrid assay according to the manufacturer's instructions. *S. cerevisiae* strain AH109 was used as the reporter strain, The indicated proteins were fused to the GAL4 activation domain in vector pGADT7 and the GAL4 DNA-binding domain in pGBKT7 and expressed in AH109. The reciprocal combinations of fusions with the activation domain and DNA-binding domain were examined. The interactions were judged by a spot test on three types of dropout (DO) plates: 4DO (SD medium lacking adenine, histidine, leucine, and tryptophan; high-stringency conditions), 3DO (4DO plus adenine; medium stringency), and the control 2DO (SD without leucine and tryptophan) to select plasmids.

Disruption of *nbs1*$^+$ gene. An Nbs1 knockout plasmid, pNT117, was constructed as follows. A 0.8-kb fragment containing the sequence upstream of the *nbs1*$^+$ ORF, which was amplified by PCR with genomic DNA and primers IWA180 (5′-CACAAGCTTGTATACACATACTTCTCCAG-3′) and IWA181 (5′-CACCTCGAGGGTTTAGTAGATTTAGCTTC-3′) was subcloned into pBluescript II SK (+), giving plasmid pNT115. A 1.0-kb fragment containing the sequence downstream of the *nbs1*$^+$ ORF, which was amplified by PCR with genomic DNA and primers IWA178 (5′-CACGGATCCGCTCTTCTTCCAAG ATTTTG-3′) and IWA179 (5′-CACAAGCTTGACTACTTACCTGAGATAT C-3′), was subcloned into pBluescript II SK(+), giving plasmid pNT114. Then the *Hin*dIII-*Bam*HI fragment containing the sequence downstream of the *nbs1*$^+$ ORF from pNT114 was inserted into the *Hin*dIII and *Bam*HI sites in pNT115, giving pNT116. Next, the 1.8-kb *ura4*$^+$ gene was inserted into the *Hin*dIII site in pNT116, giving knockout plasmid pNT117. An *Xho*I-*Bam*HI fragment carrying the *nbs1*::*ura4*$^+$ construct derived from knockout plasmid pNT117 was transformed into haploid strain SPN124 (*smt-0 ura4-D18 leu1-32 his3-D1 arg3-D1*) with the lithium acetate method (42). Stable transformants were isolated, and gene disruption was confirmed by Southern blot analysis.

Measurement of telomere length. Telomere length was measured by Southern hybridization according to the procedure described previously (13) with the AlkPhos Direct kit module (Amersham Pharmacia Biotech). Briefly, chromosomal DNA which had been digested with *Apa*I and separated by electrophoresis on a agarose gel was probed with a 0.3-kb DNA fragment containing the telomeric repeat sequences derived from pNSU70 (a gift from N. Sugawara).

RESULTS AND DISCUSSION

Newly isolated *slr* mutants. To identify novel genes involved in homologous recombination and recombination repair of *S. pombe*, we isolated *slr* mutants as described in Materials and Methods. From approximately 100,000 cells mutagenized with *N*-methyl-*N*′nitro-*N*-nitrosoguanidine, 12 colonies were identified as *slr* mutants (Table 2). Complementation analysis of MMS and UV sensitivities with the cloned genes *rhp51*$^+$, *rhp55*$^+$, and *rhp57*$^+$ revealed that a mutation of mutant 11 in Table 2 was an allele of *rhp57*$^+$. We subsequently attempted to isolate plasmids that complemented the MMS sensitivity of the 11 remaining mutants from two genomic libraries. Four genes have been isolated to date. Mutants 6, 7, and 23 were complemented by plasmids carrying the *rad32*$^+$ gene. Sequencing of the genomic DNA revealed that the *rad32*$^+$ gene was mutated in these strains. This is consistent with a previous report demonstrating that *rad32* mutations are lethal in combination with a *rad2* mutation (58).

One plasmid that complemented the sensitivity of mutant 27 was isolated (pNT101). pNT101 carried a 3.3-kb genomic

TABLE 2. Summary of *slr* mutant isolation

Mutant no.	Mutant gene[a]	*S. cerevisiae* homologue
6	*rad32*	*MRE11*
7	*rad32*	*MRE11*
11	*rhp57*	*RAD57*
14	ND	
16	ND	
17	ND	
23	*rad32*	*MRE11*
27	*slr8* (this study)	*XRS2*
28	ND	
29	ND	
34	ND	
45	ND	

[a] ND, not determined.

DNA fragment of cosmid c6B1. Since there are no known genes involved in DNA repair and recombination in this region, we named it *slr8*$^+$ (since we had already isolated seven *slr* mutants) (62) and analyzed it further in this study.

Identification of the *nbs1*$^+$ gene in *S. pombe*. Complementation analysis with several deletion plasmids derived from pNT101 revealed that *slr8*$^+$ corresponds to the open reading frame SPBC6B1.09c. The cDNA was prepared, and its sequence revealed that *slr8*$^+$ contained six introns, although only five of these were predicted in the Sanger Centre database (http://srs.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-e+[{EMBL%20EMBLNEW}-acc:AL021838]) (see Materials and Methods). RACE experiments revealed that mRNA for *nbs1*$^+$ was transcribed from 82 bp upstream from the putative initiation codon to 110 bp downstream from the predicted stop codon. Since there was a stop codon and no ATG codons in-frame upstream of the putative ATG, this should be the actual initiation codon. These cDNA analyses indicate that the *slr8*$^+$ gene product consists of 613 amino acids. Genomic DNA sequencing of the *slr8* locus revealed that the codon for amino acid Gln-88 (CAA) in Slr8 protein is mutated to a nonsense sequence (TAA) in the *slr8* mutant, suggesting that the gene is responsible for the *slr8* mutation. The features of the amino acid sequence of Slr8 (see below) suggest that this protein could be an Nbs1 homologue, and therefore we designated Slr8 Nbs1 in *S. pombe*.

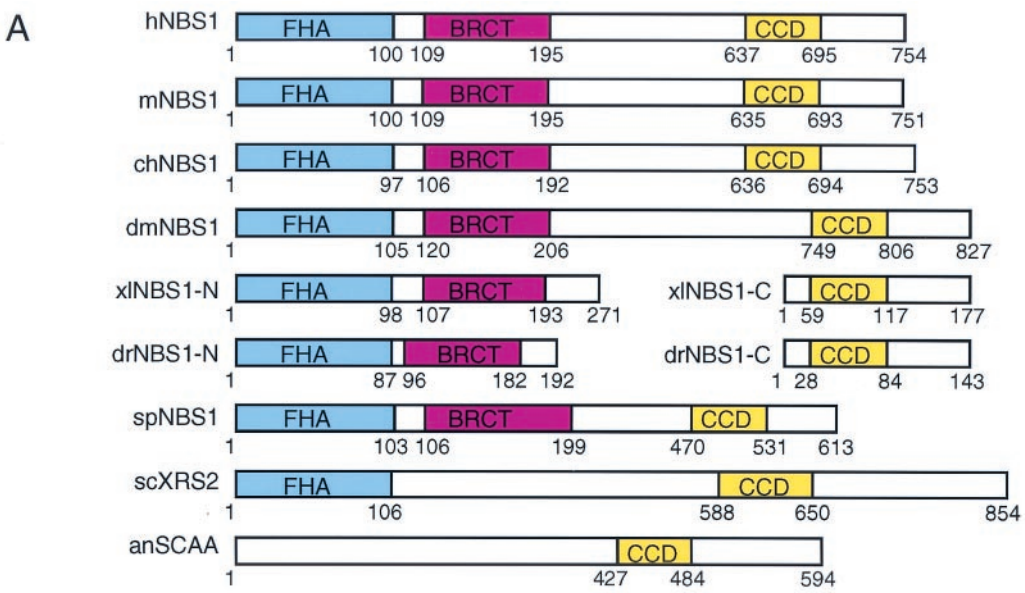
Structure of *S. pombe* Nbs1/Slr8 protein and Nbs1 homologues. Vertebrate Nbs1 proteins contain a forkhead-associated domain in the N-terminal region, followed by a BRCT domain and a C-terminal conserved domain (CCD) (Fig. 1A). The *S. pombe* Slr8 also contains a forkhead-associated domain in the N-terminal region (amino acids 1 to 103) and a CCD in the C-terminal region (amino acids 470 to 531), which show 26% and 23% identity to the forkhead-associated domain and CCD in human Nbs1, respectively (Fig. 1A, B and D).

The domain search program at the NCBI server (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps) did not detect a BRCT domain in Slr8. However, hydrophobic cluster analysis (7) revealed that Slr8 contains all five of the motifs (designated A to E) characteristic of the BRCT domain (Fig. 1C). In addition to these five motifs, the sequence corresponding to the alpha helix (α2) in the BRCT domain is highly conserved in Slr8 (Fig. 1C). This region (amino acids 106 to 199) shows 18% identity to the BRCT domain of human Nbs1 (Fig. 1C). These sequence features suggest that Slr8 contains a BRCT domain in this region. Therefore, we conclude that Slr8 is a structural homologue of Nbs1, although the overall similarity is very limited. *S. cerevisiae* Xrs2, which is considered an Nbs1 counterpart, does not seem to possess a BRCT domain (data not shown) (14). Therefore, Nbs1 is a more appropriate name than Xrs2 for the Slr8 protein.

The forkhead-associated domains in human Chk2 and *S. cerevisiae* Rad53 have been shown to bind to phosphorylated proteins (1, 53). The BRCT domain is also predicted to be a protein-protein interaction domain (69). Indeed, the recombinant fragment containing the forkhead-associated/BRCT domain of human Nbs1 bind to phosphorylated histone H2AX (γ-H2AX) (32). Therefore, *S. pombe* Nbs1 most probably binds to phosphorylated histone H2A. The physiological significance of this interaction remains unclear and will be the subject of future studies.

A database search revealed that CG6754-PB protein from *Drosophila melanogaster* contains the three domains (forkhead-associated, BRCT, and CCD), suggesting that it could be the Nbs1 homologue in the fruit fly (Fig. 1A). Moreover, two protein fragments (we designated them drNBS1-N and drNBS1-C) predicted from cDNA clones in the zebra fish (*Danio rerio*), BI984731 and BM775439, and two protein frag-

FIG. 1. Amino acid sequence analysis of *S. pombe* Nbs1. Alignment was performed as described previously (64). (A) Overall structural comparison among several Nbs1 and related proteins. (B) Forkhead-associated (FHA) domain. (C) BRCA1 C-terminal (BRCT) domain. The five conserved regions, designated A to E (7), of the BRCT domain based on the hydrophobic cluster analysis (HCA) are shown by a dotted arrow. The secondary structure of the XRCC1 BRCT domain (69) is shown at the bottom of the alignment. (D) C-terminal conserved domain (CCD). Protein sequences were obtained from humans (h), mice (m), chickens (ch), *Aspergillus nidulans* (an), *Drosophila melanogaster* (dm), *Danio rerio* (dr), *Xenopus laevis* (xl), *S. pombe* (sp), and *S. cerevisiae* (sc). Color of conserved positions (at most, two deviating amino acids for the forkhead-associated domain, four deviating amino acids for the BRCT domain, and three deviating amino acids for the CCD): green, hydrophobic (FYWILMVTAC); red, acidic (DEQN); blue, basic (HKR); magenta, glycine (G); brown, serine or threonine (ST); yellow, proline (P). Sequences are denoted by species identification prefixes for *Caenorhabditis elegans* (ce) and *Arabidopsis thaliana* (at) and by protein designations for hNBS1 (GenBank BAA28616); spNBS1 (GenBank AB099299); drNBS1-N (GenBank BI984731); drNBS1-N (GenBank BM775439); xlNBS1-N (GenBank CA988284); xlNBS1-C (GenBank BG022948); scXRS2 (GenBank AAB64805); mNBS1 (GenBank AB016988); chNBS1 (GenBank AAG47947); dmNBS1, also named CG6754-PB protein (GenBank NM_143716); predicted translation products of the cosmids F37D6.1 (GenBank CAA99847) and T10M13.12 (GenBank T01512); the CAG trinucleotide repeat containing cDNA CAGF28 (GenBank AAB91434); the BRCA-associated RING finger domain protein BBARD (GenBank Q99728); the DNA repair proteins XRCC1 (GenBank A36353) and REV1 (GenBank S67255); the oncoprotein ECT2 (GenBank S32372); the breast cancer susceptibility type 1 protein BRCA1 (GenBank U14680); the radiation-sensitive checkpoint protein Rad9 (GenBank M26049); and campothecin resistance-conferring protein SCAA (GenBank AAF81094), which has moderate structural similarity to hNBS1. Numbers in the alignment denote the number of amino acids omitted.



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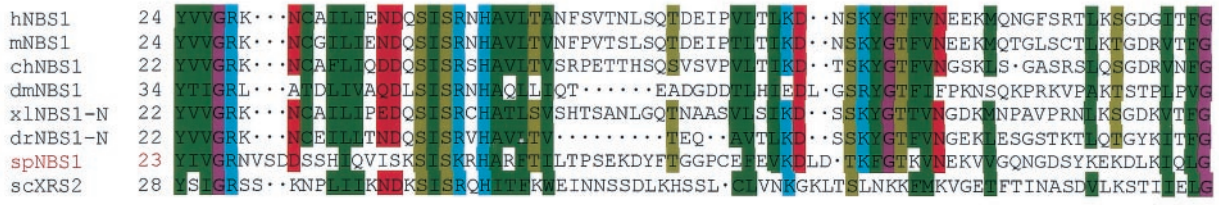

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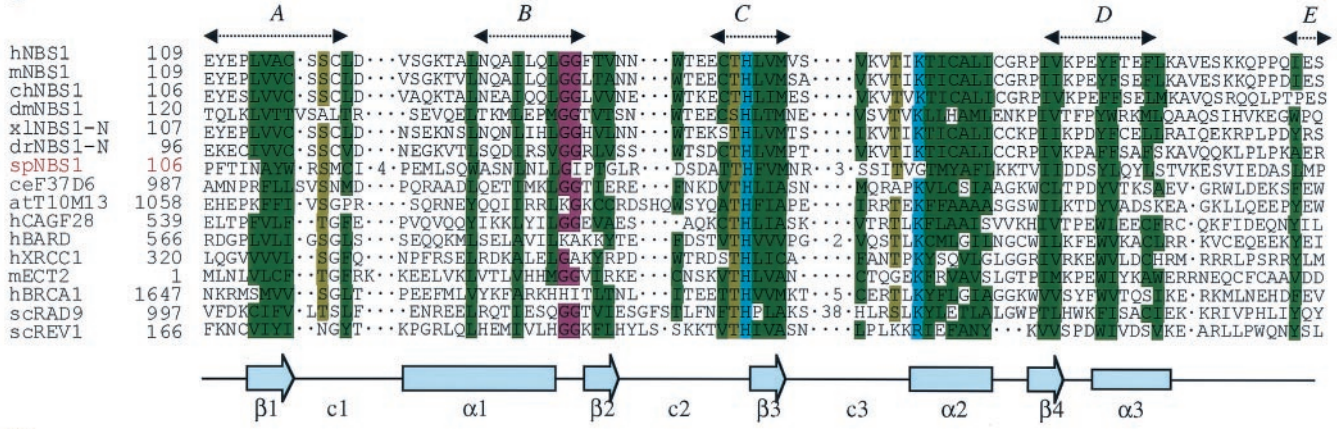

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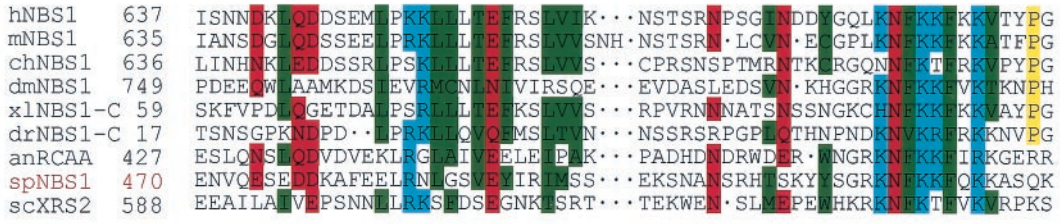

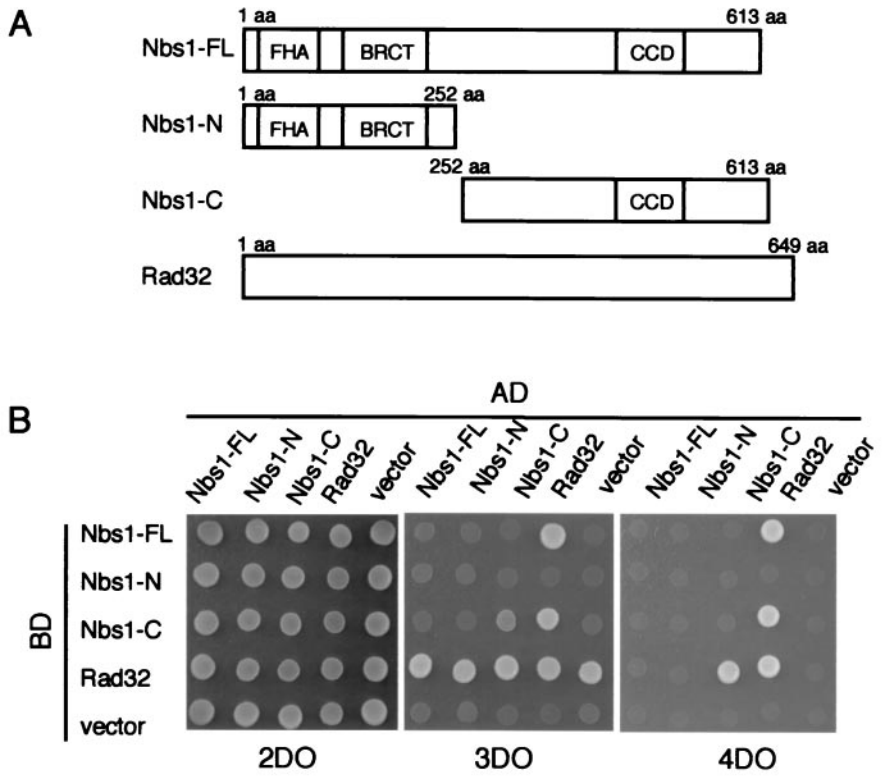

FIG. 2. Yeast two-hybrid interaction between Nbs1 and Rad32. (A) A simple scheme of the protein structure and constructs of Nbs1 designed for yeast two-hybrid analysis. Nbs1-FL, full-length Nbs1 (amino acids [aa] 1 to 613); Nbs1-N, N-terminal half of Nbs1 (amino acids 1 to 252); Nbs1-C, C-terminal half of Nbs1 (amino acids 252 to 613). Forkhead-associated domain (amino acids 1 to 103), BRCT domain (amino acids 106 to 199), and C-terminal conserved domain (CCD) (amino acids 470 to 531) are indicated. (B) Two-hybrid interaction of Nbs1 and Rad32. Pairwise interaction of pGBKT7 and pGADT7 was judged by a spot test on three types of dropout plates: 4DO (high stringency), 3DO (medium stringency), and 2DO (control) (see Materials and Methods).

ments (designated xlNBS1-N and xlNBS1-C) predicted from cDNA clones in the frog (*Xenopus laevis*), CA988284 and BG022948, showed high similarity to the human NBS1 N-terminal fragment and C-terminal fragment, respectively (Fig. 1A). These fragments contain either the forkhead-associated and BRCT domains or CCD which show high similarity to those of human Nbs1 (Fig. 1B to D). These amino acid sequence features strongly suggest that frog and zebrafish contain NBS1 proteins, although the full-length cDNAs that encode frog or zebrafish NBS1 have not been identified. These cDNA fragments containing the forkhead-associated/BRCT domains and CCD might be truncated products of the same genes.

We also found that the amino acid sequence (427 to 484) of ScaA protein in the filamentous fungus *Aspergillus nidulans*, which is suggested to be related to human NBS1 (5), shows high similarity to CCD (amino acids 637 to 695) in human NBS1 (Fig. 1D). Strains carrying the *scaA* mutation are hypersensitive to camptothecin, MMS, UV light, and γ-rays (5). The sequence of amino acids 230 to 513 of ScaA shows 20% identity and 38% similarity with that of amino acids 280 to 572 of *S. pombe* Nbs1. These facts further suggest that ScaA is most probably a functional Nbs1 homologue in *A. nidulans*, although ScaA possesses neither a forkhead-associated nor a BRCT domain.

C-terminal half of Nbs1 binds to Rad32 in a two-hybrid assay. Since human Nbs1 and *S. cerevisiae* Xrs2 physically interact with Mre11 (10, 21, 63), we tested whether *S. pombe* Nbs1 also interacts with Rad32, an *S. pombe* homologue of Mre11. A yeast two-hybrid assay was performed in which reciprocal combinations of fusions of Nbs1 and Rad32 with the GAL4 activation domain (AD) and the GAL4 DNA binding domain (BD) were examined (Fig. 2). A BD-Nbs1-FL fusion protein interacted with the AD-Rad32 fusion at high stringency (4DO). Although we did not detect interaction between the AD-Nbs1-FL and BD-Rad32 under high-stringency conditions, we did observe this at lower stringency (3DO). The inability to detect interactions with particular combinations of activation domain and DNA-binding domain fusion proteins may have been due to a very low level of expression of the fusion protein. Indeed, Western blotting analysis revealed that the protein band corresponding to AD-Nbs1-FL was barely detectable in the extract from the *S. cerevisiae* tester strain (data not shown). Notably, the two-hybrid interactions were observed between the C-terminal half of Nbs1 and Rad32 under high-stringency conditions, while the N-terminal half of Nbs1 did not interact with Rad32 (Fig. 2B). This is consistent with the proposal that Nbs1 interacts with Mre11 via its small C-terminal conserved region (56). These two-hybrid interac-

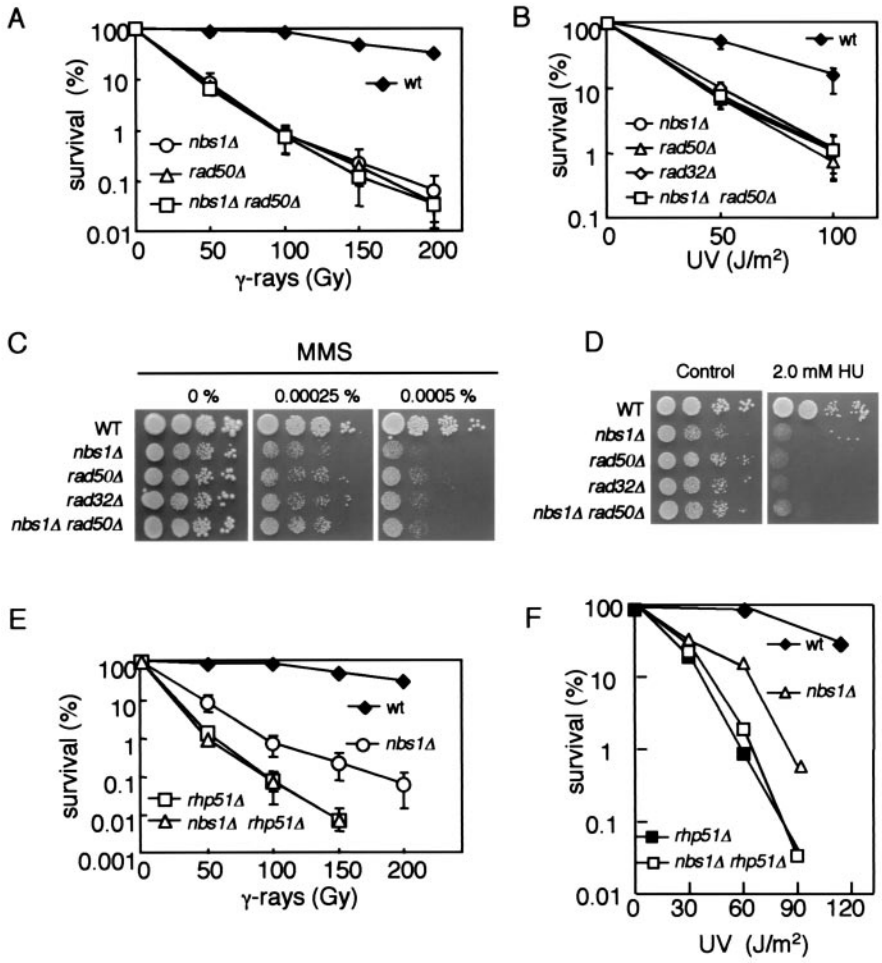

FIG. 3. DNA damage sensitivity of *nbs1*Δ cells and epistasis analysis. (A) The sensitivities to γ-ray of *nbs1*Δ cells (SPN100), *rad50*Δ cells (KT120), and *rad50 nbs1* double mutants (KW015). JY741 was used as a wild-type (wt) strain. (B) The sensitivities to UV light of *nbs1*Δ cells, *rad50*Δ cells, *rad32*Δ cells (142) and the *rad50 nbs1* double mutants. (C and D). The sensitivities of *nbs1*Δ cells, *rad50*Δ cells, *rad32*Δ cells, and *rad50 nbs1* double mutants to MMS (C) and hydroxyurea (D) determined in a spot test. (E and F). Epistasis between *nbs1*Δ cells and *rhp51*Δ cells for γ-ray (E) and UV (F) sensitivity. Wild-type (SPN124), *nbs1*Δ (SPN100), *rhp51*Δ (B54), and *nbs1 rhp51* (SPN103) cells. For genotypes, see Table 1. Standard deviations are shown by error bars.

tions between Nbs1 and Rad32 suggest that *S. pombe* Nbs1 functions together with Rad32 in vivo.

Since the CCDs are highly conserved among Nbs1/Xrs2 proteins and the conservation is much higher than that of the forkhead-associated or BRCA domains (Fig. 1D), it is suggested that the molecular mechanism of the interaction between Nbs1 and Mre11 is conserved in eukaryotes. We also suggest that the CCD could be used for searching for Nbs1 homologues in other organisms as described above.

Construction of *nbs1* disruptants. To study the in vivo function of *nbs1*$^+$ of *S. pombe*, we made a heterozygous strain (*nbs1*$^{+/-}$) with a one-step gene replacement procedure, in which one of the chromosomal *nbs1*$^+$ genes was replaced with a *ura4*$^+$ cassette. Spores derived from the heterozygote were viable regardless of auxotrophy for uracil, indicating that *nbs1*$^+$ is not essential (data not shown). However, the growth of the *nbs1* disruptant was poor compared with that of the wild-type strain. The synthetic lethality of the *nbs1* disruption with *rad2*Δ was confirmed by analyzing the segregants of *nbs1*$^{+/-}$ *rad2*$^{+/-}$ heterozygotes (data not shown). We also constructed an *nbs1* null mutant from a haploid strain as described in Materials and Methods and used it for further characterization.

DNA repair activity of *nbs1*Δ mutants. We first characterized the DNA repair activity of *nbs1* null mutants. An *nbs1* single mutant showed high sensitivity to γ-ray irradiation, indicating that Nbs1 is involved in DSB repair (Fig. 3A). In addition, the mutant was very sensitive to UV, MMS, and hydroxyurea, suggesting that Nbs1 is also involved in the repair of many types of DNA damage in addition to DSBs (Fig. 3B to D). The hydroxyurea sensitivity of the *nbs1* mutant might imply the involvement of *S. pombe* Nbs1 in the S-phase checkpoint. The sensitivities of the *nbs1* single mutant to these DNA damages were very similar to those of *rad32* and *rad50* single mutants, and combinations of these mutations with an *nbs1* mutation did not affect these sensitivities (Fig. 3A to D). These

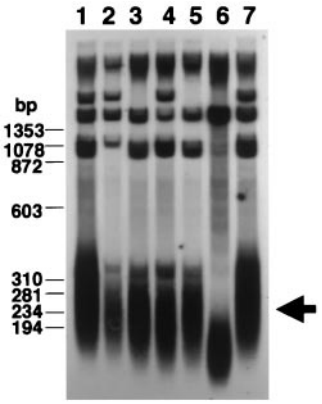

FIG. 4. Nbs1 is involved in telomere length maintenance. The telomere length of wild-type, *nbs1*Δ, *rad50*Δ, *rad32*Δ, and *rad50 nbs1* double mutant cells was evaluated by Southern hybridization. Lane 1, wild-type cells (JY741); lane 2, *nbs1*Δ (SPN100); lane 3, *rad50*Δ (KT120); lane 4, *rad32*Δ (142); lane 5, *rad50 nbs1* double mutant (KW015); lane 6, *rad3*Δ (rad3D); lane 7, wild-type cells (JY741). Genomic DNA was digested with *Apa*I and separated by electrophoresis on a 2% agarose gel. Telomeres are indicated by an arrow. For genotypes, see Table 1.

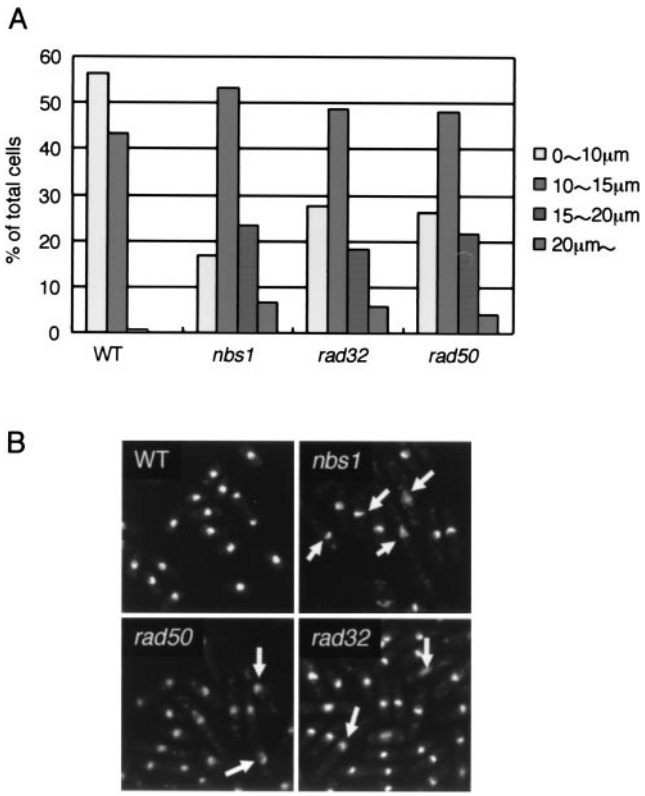

FIG. 5. *nbs1*Δ, *rad50*Δ, and *rad32*Δ cells are all elongated and exhibit abnormal nuclear morphology. (A) Cell length of wild-type (WT, SPN114), *nbs1*Δ (SPN138), *rad50*Δ (SPN148), and *rad32*Δ (SPN141) cells was determined by microscopy. (B) Nuclear morphology of wild-type, *nbs1*Δ, *rad50*Δ, *and rad32*Δ cells. The cells were fixed with 2.5% glutaraldehyde and stained with 1 μg of DAPI per ml. Fluorescence images obtained with an epifluorescence microscope are shown. Arrows indicate nuclei with abnormal morphology. For genotypes, see Table 1.

results suggest that Nbs1 functions together with Rad32 and Rad50, perhaps via a complex similar to the Mre11-Rad50-(Xrs2/Nbs1) complex.

Rhp51 protein from *S. pombe* is the functional homologue of *S. cerevisiae* Rad51, which plays central roles in the homology search and DNA strand exchange reactions during homologous recombination and recombination repair (44). The sensitivities to γ-ray and UV of an *nbs1 rhp51* double mutant were very similar to those of the *rhp51* single mutant (Fig. 3E and F). Taken together, our results suggest that *nbs1*$^+$ belongs to the same epistatic group in DNA repair as *rad50*$^+$, *rad32*$^+$, and *rhp51*$^+$ and that *nbs1*$^+$ is involved in recombination repair.

Treatment with MMS, hydroxyurea, or UV, unlike γ-ray irradiation, does not directly produce DSBs. However, the fact that *rad22*, *rhp51*, and *rhp54* mutants all have significantly increased sensitivities to these treatments indicates that recombination is very important for recovery from the damage caused by those agents in *S. pombe*. These treatments cause replication fork arrest. When replication forks are stalled, Holliday junctions or chicken-foot structures of DNA are thought to be produced, and those would either be reversed by Rqh1 or resolved by Mus81-Eme1 endonuclease (4, 35). In the latter pathway, it is thought that DNA DSBs are generated upon cleavage by Mus81-Eme1 (19). The Rad32-Rad50-Nbs1 complex might be required for the processing of these DSBs at the replication fork to initiate recombination-dependent replication. It is also possible that the Rad32-Rad50-Nbs1 complex, rather than Mus81-Eme1 endonuclease, is directly involved in the cleavage at collapsed replication forks, since it has been suggested that Rad32 cleaves the hairpin structure generated on the lagging DNA strand during S phase (20).

Nbs1 is involved in telomere length regulation. As *S. pombe* Rad50 and Rad32 are involved in telomere length regulation (25, 66) and Rad32 has been shown to associate with telomeres (47), we examined the telomere length of *nbs1* mutants. The telomeres of the *nbs1* mutant were shorter than those of the wild-type strain (Fig. 4). The telomere length of the *nbs1* mutant was almost the same as those of *rad32* and *rad50* single mutants and the *nbs1 rad50* double mutant. These results indicate that Nbs1 is required for telomere length maintenance. Recently, we found that deletion of *taz1*$^+$ significantly increased the single-stranded G-rich overhang at telomere ends and that this overhang disappeared after concomitant deletion of *rad32*$^+$, *rad50*$^+$ (58a), or *nbs1*$^+$ (data not shown). This result suggests that Nsb1 functions together with Rad32 and Rad50 at telomere ends. Human Mre11 and Rad50 bind to telomere ends in a cell cycle-independent manner, while human Nbs1 binds to telomeres only during S phase, suggesting that the MRN complex, a multisubunit nuclease composed of Mre11, Rad50, and Nbs1, plays an important role in telomere maintenance in S phase (71). Consistent with this, it has been suggested that the *S. cerevisiae* MRX complex may be required for recruitment of the telomerase components to telomeres during S phase (55, 61).

***nbs1*Δ cells are elongated and exhibit abnormal nuclear morphology.** Since the growth of the *nbs1*Δ cells was fairly poor, we attempted to observe the cell morphology by microscopy. *nbs1*Δ cells as well as *rad32*Δ cells and *rad50*Δ cells were significantly elongated (Fig. 5A). *rhp51*Δ cells and *rhp54*Δ cells

are also elongated (45). As the Rad32-Rad50-Nbs1 complex, Rhp51 and Rhp54 are all required for recombination repair, elongation of the cells may be due to the accumulation of DNA damage during normal mitotic growth. Such DNA damage would activate the DNA damage checkpoint, which would retard the cell cycle and cause elongation of the cells (9). Consistent with this, we observed a slight activation of the checkpoint kinase Cds1 in *rad50* mutants in the absence of any DNA-damaging agents (data not shown) (3).

In addition, most of the *nbs1*Δ cells had aberrant nuclear morphology, as visualized by 4′,6′-diamidino-2-phenylindole (DAPI) staining, whereas the nuclear chromosomal domain of wild-type cells was hemispherical or round (Fig. 5B). The nuclei of most *nbs1* mutant cells appeared diffuse, and a compressed and crescent-like structure was observed within them. The *rad32*Δ cells and *rad50*Δ cells also had similar aberrant nuclear morphology (Fig. 5B). These results indicate that Nbs1, probably in a protein complex with Rad32-Rad50, is required for maintaining chromosomal structure.

Perspectives. The abnormal nuclear morphology of the *rad32*, *rad50*, and *nbs1* mutants might be related to a defect in chromatin cohesion. The depletion of a component of the cohesin complex, Rad21, from cells has been shown to result in disturbance of the nuclear organization (2), and a functional link between Rad21 and Rad50 has also been suggested (25). Such a link between the cohesin complex and the MRN complex has also been suggested in mammals. For example, human SMC1 is phosphorylated in response to ionizing radiation in an Nbs1-dependent manner (31, 68). More recently, a direct interaction between the Mre11-Rad50 complex and cohesin at DNA damage sites has been reported (30). However, the exact roles of the cohesin complex in DNA repair and the maintenance of nuclear organization remain unclear. More extensive investigations of the roles of *S. pombe* Nbs1 in the DNA damage response would reveal the molecular details of the DNA damage checkpoint pathway and of the possible functional link between the Nbs1 complex and cohesin in DNA repair and the maintenance of nuclear organization.

Although both the *S. cerevisiae* MRX complex and vertebrate MRN complexes have been suggested to be involved in both DNA repair and checkpoint signaling (14), there are several differences in the roles of these complexes in DNA repair and checkpoint signaling. For example, the *S. cerevisiae* MRX complex has been reported to be partially required for phosphorylation of the Rad53 checkpoint kinase in response to DSBs (23). On the other hand, activation of human CHK2, a functional homologue of *S. cerevisiae* Rad53, in response to ionizing radiation is intact in NBS1 cells (68). In addition, the *S. cerevisiae* MRX complex is required for nonhomologous end joining repair, while chicken Nbs1 is not (40, 57). Like vertebrate MRN complexes, *S. pombe* Rad50 is not required for nonhomologous end joining repair (36). Moreover, the sequence features of *S. pombe* Nbs1 are closer to those of homologues in higher eukaryotes than to those of *S. cerevisiae* Xrs2 (Fig. 1), suggesting that the *S. pombe* Rad32-Rad50-Nbs1 complex may have functional similarity to the MRN complexes of higher eukaryotes rather than to the MRX complex of *S. cerevisiae*.

Conclusions. We succeeded in identifying the Nbs1 homologue in *S. pombe*. *S. pombe nbs1*$^+$ is required for DNA repair, telomere length regulation, and maintenance of chromatin structure. The amino acid sequence features and the DNA repair function suggest that the *S. pombe* Rad32-Rad50-Nbs1 complex has functional similarities to the MRN complexes of higher eukaryotes. Accordingly, *S. pombe* Nbs1 will become a convenient model system for studying the molecular function of the Mre11 (Rad32)-Rad50-Nbs1 complex, which plays very important roles in DNA repair, the DNA damage checkpoint, and the maintenance of telomere and chromatin integrity. In addition, it will also provide clues to understanding the molecular mechanism of cancer development observed in ataxia telangiectasia and Nijmegen breakage syndrome patients.

ACKNOWLEDGMENTS

We thank Akira Matsuura, Takashi Ushimaru, and Masahiro Uritani for discussion and sharing strains; Takeshi Saito, Shinji Yasuhira, and Hiroshi Utsumi for help with the γ-ray irradiation; and Nicholas Rhind and Paul Russell for sharing unpublished results. We also thank Masahiko Okuno and Yoshifumi Nishimura for valuable comments on the prediction of the BRCT domain. We thank Hisao Masukata for providing the *S. pombe* genomic libraries, Antony Carr for providing the *S. pombe* genomic libraries and critical reading of the manuscript, and Kenshi Komatsu for critical reading of the manuscript.

This work was supported in a part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan to H.I., H.S., and M.U., by grants from the Human Frontier Science Program Organization (HFSPO) to H.I. and H.S., and by a grant from the Yokohama City Collaboration of Regional Entities for the Advancement of Technological Excellence, JST, to M.U. Part of this work was performed with the facilities of the Research Reactor Institute, Kyoto University.

M. Ueno and H. Iwasaki contributed equally to this work.

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